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TITLE OF INVENTION:

METHOD FOR THE DETECTION AND MEASUREMENT OF HAPten-
CONJUGATED BIOLOGICAL BINDING ENTITIES BY WESTERN AND DOT-
BLOT USING ANTI-HAPten ANTIBODIES.

8 CROSS REFERENCE TO RELATED APPLICATIONS: None

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR

DEVELOPMENT: This invention was not directly supported by any federally
12 sponsored research.

REFERENCE TO SEQUENCE LISTING, TABLES, OR COMPUTER

PROGRAM LISTINGS: None

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BACKGROUND OF THE INVENTION

20 1. Frequently, researchers desire to analyze the ability of various proteins and other factors to bind to cell surfaces. Usually, the type of binding studied is one where the binding factor (ligand) recognizes and binds to a specific receptor for it on the cell surface. Thus, these types of studies are used to examine the inherent properties of the
24 ligand itself, but also are used solely to study the receptor. Analysis of ligand binding to cell surfaces is usually performed directly, wherein that ligand itself is obtained in pure

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form and is radiolabeled. Usually, ligands are radiolabeled with ^{125}I . More rarely, they are purchased labeled with ^3H or ^{14}C . The labeled ligand is assessed for its maintenance of activity, and for its specific (cpm per unit of weight) radioactivity. To measure binding , the radiolabeled material is applied under established optimal conditions to desired cells of known density (cells/unit volume or cell protein/unit volume). Typically, various concentrations (from high to low) of the ligand are added to separate tubes or dishes of cells. Certain cell containers at each dose tested also receive an excess of unlabeled pure ligand. Usually, these excesses are 10 – 200 fold times the concentration of labeled ligand. After the desired binding time has passed, the unbound material from all samples is saved and the cells are washed free of all unbound labeled and unlabeled ligand. The cells are then placed into counting tubes and counted for radioactivity.

Initial unbound material is counted also. The amount of labeled ligand bound or unbound is calculated from the known specific cpm. Counts obtained from unlabeled excess ligand-receiving samples are subtracted from the counts obtained from samples treated with like-dose labeled ligand only. This provides specific cpm bound. The weight amount of specific labeled ligand bound is calculated from the known specific cpm per unit weight. Knowing the cell density, one can calculate amount of specific ligand bound per cell at each ligand dose level. Usually, the data is plotted as specific ligand bound/ligand unbound/ unit of cells on the y axis and specific ligand bound/unit of cells on the x axis. This produces data with a negative slope and the x-intercept is the maximum amount of ligand able to bind. Therefore, the x-intercept also represents the

receptors/cell for the ligand (Scacchi *et. al.*, 1988; Inoue *et. al.*, 1993; LaGrange *et. al.*, 1993; Cavanaugh and Nicolson, 1998; Cavanaugh *et. al.*, 1999).

4 2. An alternative method to determine ligand binding to cells is to conjugate a particular fluorescent molecule to the pure ligand. Fluorescent labeled material is allowed to bind to cells at various concentrations with or without the presence of unlabeled ligand. After binding is complete, all unbound ligand is washed off and the
8 fluorescence of the cells is determined using a fluorescent spectrophotometer or a fluorescent activated cell sorter (FACS) instrument (Chaudary, *et. al.*, 1994; Gordon, 1995; Niedergang *et. al.*, 2000; Palupi *et. al.*, 2000). This procedure is more difficult to standardize and precise quantitation of ligand receptors/cell is not as accurate as with
12 Scatchard analysis using radiolabeled ligand. This method is more given to comparing binding capacity between two different cell populations. With FACS, it also requires that the binding surface exist in a monodispersed state capable of being analyzed in the flow cell of that instrument.

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3. It is also possible to allow ligand binding to cell surfaces and to then incubate the cells with a fluorescent labeled antibody to the ligand, wash, and analyze cell fluorescence by fluorescent spectrophotometry or FACS. To assess ligand receptor levels
20 only, one can incubate cells with a fluorescent labeled antibody to the receptor and measure the fluorescence of the cells by FACS (Cavanaugh and Nicolson, 1998; Cavanaugh *et. al.*, 1999).

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4. Western blotting is a technique where cell lysates obtained by detergent treatment are separated by electrophoresis and the separated components contained
4 within the electrophoresis gel are driven onto a protein-binding membrane via electric current. The membrane with its cell constituents separated by molecular weight is blocked with a non-specific protein and can than be analyzed for particular cellular constituents by treatment with an antibody to that constituent followed by treatment with
8 an enzyme conjugated antibody to the first antibody. Enzyme containing regions of the membrane are detected using color-producing or light-emitting substrates for that enzyme.

12 5. Dot-blotting or slot-blotting is where the cell lysate is applied directly to a binding-membrane without prior separation by electrophoresis. The membrane is blocked and treated as described in the previous paragraph to detect particular cell constituents. Unlike Western-blotting, the molecular weight of detected material is not
16 ascertained.

6. We found that the binding of transferrin to tumor cell surfaces correlated with the aggressiveness of those cells; i.e.: the more metastatic tumor cells bound more
20 transferrin than did poorly metastatic cells (Cavanaugh and Nicolson, 1991, Cavanaugh and Nicolson, 1998; Cavanaugh *et. al.*, 1999). These studies required that we accurately assess the transferrin binding capability of cells in question. Initially, this was performed

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by examining the ability of the cells to bind ^{125}I -transferrin and the ability of non-labeled transferrin to inhibit that. Dealing with radioactive iodine has many drawbacks including the inherent hazardous nature of the material, its short shelf life, and expensive waste disposal. In searching for novel methods for measuring transferrin binding using non-radioactive procedures, we came upon the discovery that fluorescein-labeled (or FITC-labeled) transferrin (hereafter referred to as FITC-Tf) would stimulate the growth of cells in culture similarly to native transferrin. We also found that FITC-Tf could be internalized by cells and that this internalization could be competed for by an excess of un-labeled (or native) transferrin. The apparent retention of biological activity by FITC-Tf lent us to examine other technologies available to specifically detect the labeled protein. Many antibody suppliers now sell anti-FITC antibodies. These were initially developed to detect FITC-labeled oligonucleotides hybridized to sample RNA on Northern blots. These same antibodies can easily detect FITC-labeled proteins on Western blots (Samuel *et. al.*, 1988). We next assessed as to whether or not the combination of these reagents together would allow for the detection of FITC-labeled protein bound to cell surfaces. Cells were treated with FITC-Tf with and without an excess of native Tf. After an appropriate incubation period, the cells were washed extensively and lysed with a detergent containing buffer. The lysate components were separated by electrophoresis and electroblotted onto a nitrocellulose membrane. The membrane was blocked with non-fat dry milk and incubated with a rabbit anti-FITC antibody. The membrane was washed and incubated with goat horse radish peroxidase (HRP)-conjugated anti-rabbit IgG. The membrane was washed again and treated with a

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light emitting (enhanced luminescence) substrate for HRP. One band at \approx 70,000 in molecular weight was seen in all lanes loaded with cells that initially were exposed to FITC-Tf only. In lanes loaded with cells that had also received an excess of native Tf, a 4 markedly reduced band, or no band at all was seen. This method allowed for the sensitive determination of Tf binding to cells without the need for radioactively labeled Tf. Furthermore, the molecular weight of the bound ligand was verified via the electrophoresis step.

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7. The major difference in the method of this invention in comparison to those of the referenced patents and literature papers is the final detection method. In our case, the bound hapten-ligand is detected by immunological means after solubilization (or cell 12 lysis) and immobilization onto a membrane. In the referenced cases, bound radio-labeled ligand is detected on solubilized cells by counting; or in the case of fluorescent-labeled ligands, by fluorescent detection of the label on intact cells by optical means such as cytometry.

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BACKGROUND REFERENCES:

20 U.S. Patent Documents:

5,576,436	November, 1996	McCabe et. al.
6,054,557	April, 2000	Faure

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Specific Documents:

- Cavanaugh, P.G. and Nicolson, G. L. "Lung derived growth factor that stimulates the
4 growth of lung-metastasizing tumor cells: Identification as transferrin", Journal of
Cellular Biochemistry, 47:261-271, 1991.
- Cavanaugh, P.G., and Nicolson, G.L., "The selection of a metastatic rat mammary
adenocarcinoma cell line from a low metastatic parental population by an in vitro
8 process based on cellular ability to proliferate in response to transferrin", Journal of
Cellular Physiology, 174: 48-57, 1998.
- Cavanaugh, P.G., Jia, L., and Nicolson, G.L. , " Transferrin receptor overexpression
enhances transferrin responsiveness and the metastatic growth of a rat mammary
12 adenocarcinoma cell line", Breast Cancer Research and Treatment, 56:203-217,
1999.
- Chaudary, S., Das, S., Banerjee,S.K.,, and Sarker, P.K. "Identification and
characterization of a tubulin binding protein in rat brain plasma membrane",
16 Neurochem Int, 1994, 24, 289-299.
- Darzynkiewicz Z, Bedner E, Traganos F, Murakami T, "Critical aspects in the analysis of
apoptosis and necrosis", *Hum Cell*, 1998, 11,3-12.
- Gordon, I.L. "Scatchard analysis of fluorescent concanavalin A binding to
20 lymphocytes", Cytometry, 1995, 20, 238-244.
- Inoue, T., Cavanaugh, P. G., Steck, P. A., and Nicolson, G. L., "Differences in
transferrin response and numbers of transferrin receptors in rat and human mammary

- carcinoma lines of different metastatic potentials”, Journal of Cellular Physiology, 156: 212-217, 1993.
- Lagrange JL, Francoual M, Formento JL, Del Guidice P, Bensadoun RJ, Teissier E,
- 4 Ettore F, Gillet JY, Namer M, Milano G, “Demonstration and characterization of EGF receptors in cancer of the uterine cervix”, Bull Cancer, 1993, 80, 219-224.
- Le Gall M, Chambard JC, Breittmayer JP, Grall D, Pouyssegur J, Van Obberghen-Schilling E, “The p42/p44 MAP kinase pathway prevents apoptosis induced by
- 8 anchorage and serum removal”, Mol Biol Cell, 2000, 11, 1103-1112.
- Niedergang F, Alcover A, Knight CG, Farndale RW, Barnes MJ, Francischetti IM, Bon C, Leduc M, “Convulxin binding to platelet receptor GPVI: competition with collagen related peptides” Biochem Biophys Res Commun, 2000 273, 246-250.
- 12 Palupi NS, Franck P, Guimont C, Linden G, Dumas D, Stoltz J, Nabet P, Belleville-Nabet F, Dousset B, “Bovine beta-lactoglobulin receptors on transformed mammalian cells (hybridomas MARK-3): characterization by flow cytometry”, J Biotechnol, 2000, 78, 171-184.
- 16 Samuel D, Patt RJ, Abuknesha RA, “A sensitive method of detecting proteins on dot and Western blots using a monoclonal antibody to FITC”, J Immunol Methods, 1988, 107, 217-224.
- Scacchi GE, Turyn D, Dellacha JM, “Identification of insulin binding sites in isolated
- 20 cells from rat submaxillary gland”, Arch Biol Med Exp (Santiago), 1988, 21,189-193.

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Zhang G, Gurtu V, Kain SR, Yan G, "Early detection of apoptosis using a fluorescent conjugate of annexin V", *Biotechniques* 1997, 23,525-531.

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BRIEF SUMMARY OF THE INVENTION:

1. The present invention relates to the need in biological research to measure the ability of cells or other surfaces to bind a given compound (hereafter referred to as a ligand). The ligand could be a growth factor or any other factor whose study involves the need for persons to assess the ability of cells, or any other insoluble particle or material, to bind it. The invention requires that the binding factor be conjugated with an immunological reactive hapten such as fluorescein and at the same time retain biological and binding activity.
2. The invention makes use of many available anti-hapten antibodies which specifically recognize a hapten-conjugated binding entity or ligand (hapten-ligand) in a complex mixture of other compounds which are naturally devoid of the hapten. The hapten-ligand is presented in excess to the substrate to which it binds. After binding, excess hapten-ligand is washed off, and all bound hapten-ligand is solubilized with or without solubilized substrate components. The solubilized mixture is applied to a membrane support directly or is separated by electrophoresis and then applied to a membrane support. The included membrane-bound hapten-ligand is detected by treatment of the membrane with anti-hapten antibody and then by an enzyme-conjugated antibody to the anti-hapten antibody. The amount of resultant membrane-associated localized enzyme is determined by incubation with a color or light-producing substrate for that enzyme. For maximum sensitivity, a light-producing substrate is applied and the

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enzyme is detected by enhanced chemi-luminescence (ECL). Known amounts of pure hapten-ligand can be applied to the membrane support and similarly detected to determine a signal to dose relationship which can be used to ascertain the amount of

4 hapten-ligand in the unknowns. Thus, the system lends itself to very precise and user-defined standardization. The two-antibody incubation steps amplify the signal so that in combination with ECL, very low levels of hapten-ligand can be detected. When used this way, the system can be used to measure ligand binding to cell surfaces without the need

8 for radio-labeled ligand. Another feature of the system is that all of the reagents required are stable and have long shelf-lives. The system is a low-cost, non-hazardous, sensitive, non-radioactive, precisely standardized method for determining the binding of compounds to substrates. In particular, the method lends itself to the measurement of hapten-

12 conjugated protein binding to cell and tissue surfaces. Specifically, the method has been perfected for the use of measuring fluorescein-conjugated transferrin and fluorescein-conjugated concanavalin A binding to tissue culture cell surfaces. This invention not only offers a novel non-radioactive method for assessing ligand binding to cell surfaces,

16 but can be used to quantitate the binding of any immunologically-recognizable hapten-containing binding factor to any surface, providing that the factor can be subsequently removed, (and perhaps separated by electrophoresis; optional), and bound to a membrane support.

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BRIEF DESCRIPTION OF THE DRAWINGS:

- Figure 1.** A.) A schematic of the strategy of the assay. A cell monolayer is exposed to a
4 solution of FITC-Tf (1) or FITC-Tf plus an excess of unlabeled Tf (2). In either case, 3
molecules of Tf bind per cell. When washed and lysed, cells from 1 produced a lysate
containing 9 molecules of FITC-TF whereas cells from 2 produce a lysate containing 1
molecule of FITC-Tf. In B.), both samples are electrophoresed and the gels blotted.
8 With the cells from 1, 9 molecules of FITC-Tf are present in the 70,000 molecular weight
region of the blot; when this blot is incubated with rabbit anti-FITC and then with goat
anti-rabbit-IgG-HRP, a large band is seen. With the cells from 2, only one molecule of
FITC-Tf is present on the blot and a minimal band is seen on the blot after ECL.

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- Figure 2.** Reproductions of actual enhanced chemiluminescence films obtained when this
assay was performed (procedures described on pages 8 and 9). (A.) Measurement of
FITC-transferrin (FITC-Tf) binding to MTLn2/TfR cells. Cells at 50 – 60 % confluency
16 growing in 12 well plates were serum-starved, then treated at 4° C with increasing levels
of FITC-Tf. After 2h, cells were washed, lysed, and equal cell equivalents were
electrophoresed, blotted, incubated with goat anti-FITC, then with anti-goat-HRP and an
HRP ECL substrate. The blot was then analyzed using the BIO-RAD fluor-S
20 Multiimager. Lanes 1-6 = lysates from cell exposed to the concentration of FITC-TF
listed below the blot. Lanes 7-12 = lysates from cells treated as in 1-6, but also with a
200-fold excess of unconjugated Tf. (B.) Results from a blot treated as in A but loaded

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with pure FITC-TF standards in the amounts (in ng) indicated on the top. The pure FITC-Tf samples were electrophoresed, blotted, and measured using the two antibodies mentioned in A, followed by ECL. (C.) Results from the quantitation of B using the Bio-
4 Rad Multiimager, indicating the type of standard curves achievable.

Figure 3. A.) A schematic of the strategy of the assay when used to detect apoptotic cells. Cells in early apoptosis are known to bind the protein Annexin V whereas normal
8 cells bind little or none of this protein. Cells in apoptosis (1) or normal non-apoptotic cells (2) are exposed to a solution of FITC-Annexin V. When washed and lysed, cells from 1 produced a lysate containing FITC-Annexin V whereas cells from 2 produce a lysate containing no FITC-Annexin V. In B.), both samples are electrophoresed and the
12 gels electro-blotted. With the cells from 1, the FITC-Annexin V molecules are present in the 33,000 molecular weight region of the blot. When this blot is incubated with anti-
FITC and then with anti-goat-IgG-HRP, HRP is localized to the 33Kd region of the blot and the HRP-containing bands are detected on X-ray film using an HRP chemiluminescent
16 substrate. This produces a band on the film at 33 Kd. With the cells from 2, no FITC-Annexin V is present on the blot, the initial antibody and therefore the second antibody do not bind, no light is produced upon incubation with an HRP chemiluminescent substrate, and no band is seen on the film.

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Figure 4.) Results obtained when this assay was used to measure the binding of FITC-Annexin V to rat MTLn3 mammary adenocarcinoma cells, as described in Figure 3. The

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cells were grown to confluence in six well plates. Cells were induced to apoptose by treatment with 4 ug/ml Camptothecin (dissolved in DMSO). Controls received DMSO only. After 24h, wells were washed three times with and equilibrated in 1 ml binding buffer (25 mM HEPES, .15 M NaCl, 2.5 mM CaCl₂, pH 7.5). FITC-Annexin V was added to 50 ng/ml and the cells were incubated for 30 min at 25° C. Cells were then washed extensively with binding buffer , and lysed in 1 ml of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants were separated by SDS-PAGE. Also run on the same gel were increasing levels of pure FITC-Annexin V. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and then incubated with rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL. A scan of the hyperfilm is shown in A. Results of quantification of the standards is shown in B. The curve from B was used to calculate Annexin V bound by the cells, the results of which are shown in C. The results indicate greater Annexin V binding by the camptothecin treated cells.

Figure 5. A.) A schematic of the strategy of the assay when used to detect specific PCR products. biotin-labeled PCR products are produced from sample DNA using biotin-labeled primers. De-natured products are applied to a well which contains a bound DNA probe which is designed to recognize the middle region of the desired PCR product. The PCR products bind to the probe, the well is washed of unbound components and the hybridized PCR species removed by heat denaturation. B.) The removed products are separated by agarose electrophoresis and are blotted to a nytran membrane. The

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membrane is blocked, and incubated with rabbit anti-biotin. The membrane is washed, incubated with goat anti-rabbit IgG-HRP, washed again, and HRP containing bands are detected using a light-emitting HRP substrate and ECL. Any non-specific PCR products
4 which have made it this far can be dis-regarded, as long as they of different molecular weight from the desired product.

Figure 6.) Results obtained when this assay was used to measure the binding of FITC-Concanavalin A (Con A) to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. The growth media was replaced with a binding buffer consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Con
8 A was added to replicate wells so that the final concentrations of FITC-Con A were 0.1, 1.0, and 10.0 ug/ml. One well of each FITC-Con A concentration also received 200 ug/ml of native (un-conjugated Con A). The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 800 uL of RIPA lysing solution. The lysates were
12 centrifuged at 5,000 X g for 5 min., and the supernatants were separated by SDS-PAGE. Also run on the same gel were increasing levels of pure FITC-Con A. Separated proteins
16 were blotted onto a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL. A scan of the hyperfilm is shown in A. Results of quantification of the standards is shown in B. The curve from B was used to calculate specific Con A bound
20 by the cells, the results of which are shown in C.

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Figure 7.) Results obtained when the samples from figure 5 were analyzed by a dot blot procedure. For the standards, increasing volumes (2, 4, 8, and 16 uL) of a 100 ng/ml

- 4 FITC-Con A solution were applied to a nitrocellulose membrane. For the lysates, 4 uL of
lysates from cells treated with 0.1, 0.5, and 1.0 ug/ml FITC Con A (with or without an
sxcess native Con A) were applied to the membrane. The membrane was blocked,
incubated with rabbit anti-FITC, then with goat anti-rabbit IgG-HRP, and HRP-
8 containing sites detected with ECL (A, B). The dots were quantitated using a Bio-Rad
Fluor S imager. Data from the standards (A, C) were used to determine the amount of
Con A bound by the cells (B, D). This displays the usefulness of the technique in a dot-
blot procedure, where the SDS-PAGE and electroblottin steps are eliminated.

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- Figure 8.)** Results obtained when this assay was used to measure the binding of FITC-Avidin to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to
confluence in six well plates. The growth media was replaced with a binding buffer
16 consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as a
carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-
Avidin was added to replicate wells so that the final concentrations of FITC-Avidin were
0.1, 0.2, and 0.4 ug/ml. The cells were incubated for 2h at 4° C, washed extensively with
20 PBS, and lysed in 1 ml of RIPA lysing solution. The lysates were centrifuged at 5,000 X
g for 5 min., and the supernatants were separated by SDS-PAGE. Also run on the same
gel were increasing levels of pure FITC-Avidin. Separated proteins were blotted onto a

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nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL. A scan of the hyperfilm is shown in A. Results of quantification of the standards is shown in B.

- 4 The curve from B was used to calculate specific Con A bound by the cells, the results of which are shown in C.

Figure 9.) Results obtained when this assay was used to measure the binding of FITC-

- 8 Insulin to human K562 erythroleukemia cells. Logarithmically growing cells in suspension culture were collected by centrifugation and washed twice by suspension in and centrifugation from a binding buffer consisting of alpha-MEM containing 5 mg/ml BSA and 25 mM HEPES (pH 7.5). Cells were adjusted to a density of 2×10^6 /ml (in
12 binding buffer), and were equilibrated to 4° C. To 1 ml of cell suspension was added 20 µl of 1mg/ml FITC-Insulin (in binding buffer; final concentration = 20 µg/ml). An additional tube also received non-conjugated Insulin at a level of 200 µg/ml. Cell suspensions were incubated for 2h at 4° C while rotating slowly, and the cells were
16 collected and washed three times by suspension in and centrifugation from binding buffer. Cell pellets were lysed in 0.4 ml/tube of Schager-Von Jagow (SVJ) electrophoresis system treatment solution and treated at 95° C for 5 min. Lysates (100 µl each) were separated by SDS-PAGE run according to Schager-Von Jagow along with
20 FITC-Insulin standards, and treated aliquots of the cell-unbound incubation mixture. Gel components were transferred to nitrocellulose and membrane-associated FITC detected as described with other ligands. In the figure, a scan of the ECL film is shown.

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Increasing signal is returned for increasing loads of FITC-insulin in the standards (lanes 1-3). The FITC-insulin bound by the cells is easily observed (lane 4), and this is reduced significantly when excess un-conjugated insulin was present (lane 5). This procedure

4 consistently displays higher molecular weight forms of insulin formed after application to cells, perhaps due to the presence of insulin binding proteins (lanes 4-7).

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DETAILED DESCRIPTION OF THE INVENTION:

1. The object of the present invention is to provide a method for the sensitive non-
4 radioactive assessment of ligand binding to insoluble surfaces. Specifically, the method
developed measures the binding of transferrin, concanavalin-A, avidin, annexin-V, and
insulin to cell surfaces. The basic detailed method using fluorescein-conjugated
transferrin as a detectable antibody-recognizable hapten tracer follows. Specific
8 alterations of this procedure for other ligands are described in the description of figures
section.

2. Fluorescein-conjugated iron-saturated (holo) human transferrin was obtained from
12 commercial sources. Cultured cells to be measured were grown to 50 – 60 % confluence
in 12 well plates. Cells were incubated with serum-free minimal essential media (alpha
modification; α -MEM) for 12 h and then again with fresh α -MEM for another 12 h. The
cell number in three wells was determined by trypsinization of those cells followed by
16 enumeration on a Coulter Counter. Media in remaining wells was replaced with 1 ml
binding buffer (BB) which consisted of: 25 mM HEPES in α -MEM containing 2mg/ml
of bovine serum albumin (BSA); pH 7.5. The cell wells were then allowed to equilibrate
to 4°C in a refrigerator. Sets of 5 replicate wells received increasing amounts of FITC-
20 Tf, from 0.02 to 0.5 μ g/ml final FITC-Tf. Two wells of each FITC-Tf concentration set
then received unlabeled holo human transferrin so that the final [Tf] = 100 μ g/ml. After
a 2h incubation at 4°C, all media was saved (= unbound samples), and the wells were all

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washed 4 times by the addition and drainage of 1 ml of 4°C PBS. All wells then received 0.5 ml of an RIPA cell lysing solution which consisted of PBS containing 1% v/v NP-40, 0.5% v/v deoxycholic acid, 0.1% v/v SDS, 100 µg/ml phenylmethyl sulfonyl chloride, and 0.1 TIU/ml Aprotinin. Cells were incubated with the lysing solution for 30 min at 4°C and all lysates were pipetted into separate 1.5 ml conical tubes. The tubes were centrifuged at 5,000 X g for 10 min and 400 uL of each supernatant was transferred to a fresh tube. All of these tubes received 166 uL of a 4X concentrate SDS-PAGE treatment solution, and were treated at 95°C for 10 minutes.

3. Treated samples were loaded onto a 12 X 12 cm 10% acrylamide SDS-PAGE electrophoresis gel (150 uL/sample) and electrophoresed at 40 mA constant current until 12 the dye front was 1 cm from the bottom of the gel. The gel was equilibrated in a transfer buffer of 48 mM Tris, 39 mM glycine. A 14 X 14 cm nitrocellulose membrane was equilibrated in transfer buffer and the gel and membrane assembled into a transfer apparatus and immersed in transfer buffer. Gel components were transferred to the 16 membrane at a constant voltage of 40 V for 1.5h.

4. The membrane was blocked at 4°C overnight in a block solution consisting of Tris buffered saline (TBS: 25 mM Tris, 0.15 M NaCl, pH 7.8) containing 0.1% tween 20 and 5% w/v non-fat dry milk. The membrane was incubated with 1:1000 rabbit anti-FITC in block solution for 2h at 25°C, and washed three times (20 min each) with 50 ml TBS. The membrane was incubated with 1:2000 HRP-conjugated goat anti-rabbit IgG

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for 2h at 25°C and washed again. Each membrane was covered with an ECL substrate for HRP (Amersham), was wrapped in plastic, and was loaded into an X-ray film cassette along with an 8 X 10 inch piece of Amersham ECL hyperfilm. The film was
4 developed after 1 min exposure and an additional film was added which was developed after 20 min exposure. Bands produced on the film were quantitated using a Bio-Rad Multi Imager.

8 5. Unbound samples are run similarly to cell lysate samples. Typically, these have to be diluted 1:10 – 1:100 in SDS-PAGE treatment solution prior to electrophoresis, to produce a signal within a readable range. The assay is standardized by loading known amounts of pure FITC-Tf onto an electrophoresis gel and repeating all of the above
12 procedures. The signal returned from the imager is plotted against the amount of FITC-Tf contained in the band and a standard curve is constructed to calculate the amounts of FITC-Tf bound by the cells.

16 6. General applications: The assay strategy can apply to any ligand conjugated with a compound which can be specifically recognized by an antibody. In particular, anti-digoxigenin, anti-rhodamine and anti-biotin antibodies exist which would recognize ligands conjugated with those compounds. The material to which the ligand binds to can
20 be other than cells. Any particles or other insoluble material can serve as the binding surface. Centrifugation and re-suspension of suspended particulate binding substrates would serve as a method for washing those of unbound ligand. The current method

requires that the bound ligand be removed from the binding surface so that it can be separated by electrophoresis. It must also bind to a conventional transfer membrane for detection with the antibody. Other specific applications accomplished to date include the

- 4 study of the binding of FITC-conjugated concanavalin A to cells, the study of the binding of Avidin to cells, and the study of the binding of Annexin-V to cells. With the latter protein, this assay could be utilized to assess cellular apoptosis without the need for a FACS analyzer.

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7. The binding of annexin V to cell surfaces has been recognized as an indicator of early apoptosis (Zhang *et. al.*, 1997). With conventional procedures, cells are removed from plates, treated with FITC-annexin V, and analyzed by FACS. The removal of cells
12 from tissue culture plates using conventional trypsin or EDTA reagents can in itself induce cell stress, apoptosis, and cellular annexin V binding (Darzynkiewicz *et. al.*, 1998; LeGall *et. al.*, 2000). Therefore, the conventional use of annexin V binding as a measure of apoptosis in adherent cells is problematic. In contrast, this invention would
16 measure the binding of FITC-annexin V to adherent cultured cells *in situ* (Figures 3 and 4), where binding and washing occur first, before the cells are removed from plates for analysis. Therefore, the amount of FITC-annexin V detected would accurately represent that bound by cells in their natural culture environment. Thus, the method outlined in
20 this invention circumvents conventional problems and provides for a more authentic measure of natural cellular annexin V binding.

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8. The assay could be used to verify the hybridization of biotin-labeled DNA to other DNA molecules. In one scenario, biotin-labeled PCR products are hybridized to an immobilized DNA probe which specifically recognizes the desired PCR product (among
4 a mixture of non-specific products). After binding, the bound PCR product is released through heat de-naturation, is separated by agarose electrophoresis, electro-blotted to nytran, and is detected by incubation with species-x anti-biotin followed by incubation with anti-species-x IgG-HRP and ECL. The final result yields a major band at the
8 expected bp size of the PCR product. Any non-specific bands of different size can be ignored during analysis of the film by an image analyzer (Figure 4). As with the above stated protein procedures, the proper molecular weight of the desired product is verified.

12 9. The replacement of electrophoresis with dot-blot techniques is possible. This would require that the only immune-recognizable conjugated component present prior to dot-blotting would be the desired product and/or absolutely minimal interaction of either antibody with non-specific sample components

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10. Conclusion: the invention is a procedure for measuring the binding of an entity (ligand) to a surface by using a hapten-conjugated version of the ligand (hapten-ligand), where the hapten is recognizable by an antibody. An excess of the hapten-ligand is
20 presented to the binding surface and excess (unbound) hapten-ligand is washed off. Bound hapten-ligand is then solubilized (removed) and applied to a membrane support or separated by electrophoresis and applied to a membrane support. The membrane-bound

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hapten-ligand is detected by application of an enzyme-conjugated antibody to the hapten; or by application of an antibody to the hapten followed by application of an enzyme-conjugated antibody to the anti-hapten antibody. The resultant membrane-associated
4 enzyme is detected and quantitated by the application of a color or light-producing substrate which reacts with the enzyme. This assay method has the advantages of providing verification of the molecular weight of the binding substance (ligand) via the electrophoresis step. It eliminates the need for radioactive materials. The procedure
8 provides for high sensitivity detection as the dual antibody incubation steps amplify the signal significantly. The procedure allows for easy standardization as different user-definable levels of a standard solution of the Hapten-ligand can be simultaneously applied to the electrophoresis gel or to the dot-blot or slot-blot membrane

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